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SPOTLIGHT REVIEW

Zebrafish as a model to study cardiac development and human cardiac disease

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Abstract Over the last decade, the zebrafish has entered the field of cardiovascular research as a new model organism. This is largely due to a number of highly successful small- and large-scale forward genetic screens, which have led to the identification of zebrafish mutants with cardiovascular defects. Genetic mapping and identification of the affected genes have resulted in novel insights into the molecular regulation of vertebrate cardiac development. More recently, the zebrafish has become an attractive model to study the effect of genetic variations identified in patients with cardiovascular defects by candidate gene or whole-genome-association studies. Thanks to an almost entirely sequenced genome and high conservation of gene function compared with humans, the zebrafish has proved highly informative to express and study human disease-related gene variants, providing novel insights into human cardiovascular disease mechanisms, and highlighting the suitability of the zebrafish as an excellent model to study human cardiovascular diseases. In this review, I discuss recent discoveries in the field of cardiac development and specific cases in which the zebrafish has been used to model human congenital and acquired cardiac diseases.

Keywords Zebrafish • Heart • Development • Disease • Cardiomyopathy

This article is part of the Spotlight Issue on: Cardiac Development

1. Introduction

The zebrafish offers several distinct advantages as a genetic and embryonic model system to study cardiovascular disease. One major advantage is that zebrafish are vertebrates that can be used for unbiased and whole-genome forward genetic screens. This allows the identification of novel genes that are required for cardiovascular development. In addition, the function of unknown genes, identified by expression or interaction screens, can be investigated by various reverse genetic approaches. Zebrafish embryos are particular well suited for studying gene function during cardiovascular development, because zebrafish embryos are not completely dependent on a functional cardiovascular system for their development. In zebrafish embryos that lack blood circulation, oxygen can still enter the embryos and reach all tissues by passive diffusion owing to the small size of the embryo. This feature permits the embryos to survive the initial phase of embryonic development and allows the analysis of embryos with severe cardiovascular defects. This is in contrast to avian and mammalian embryos, which die rapidly in the absence of a functional cardiovascular system. Besides the ease of identifying and studying genetic mutants, the optical transparency of zebrafish embryos has given new insights into various cellular aspects of cardiovascular development. The combination of new transgenic technologies to drive tissue-specific expression of fluorescent proteins with sophisticated imaging technologies, such as live imaging using single- or multi-photon confocal microscopy and single-plane illumination microscopy, has steadily increased interest in the zebrafish embryo for the study of cardiovascular development and disease.

In the first part of this review, I address in chronological order the cellular and molecular events that lead to cardiogenic specification and differentiation, heart tissue morphogenesis and cardiac function. In the second part, I discuss specific cases in which the zebrafish has been used to model human congenital and acquired cardiac diseases.

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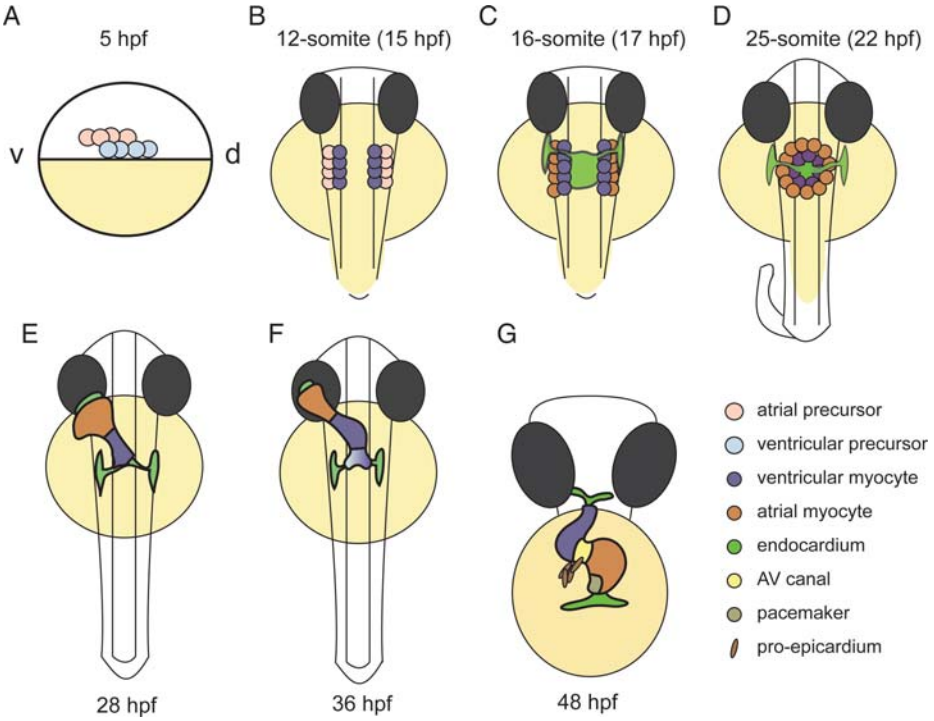

Figure 1 Stages of cardiac development. (*A*) At 5 h post fertilization (hpf) the blastula (white) covers approximately 50% of the large yolk cell (yellow). At this stage, cardiac progenitor cells are located bilaterally in the lateral marginal zone. Atrial progenitor cells (pink) are located more ventrally than the ventricle progenitor cells (light blue). During gastrulation, the cardiac progenitor cells move dorsally towards the mid-line to end up in the anterior later plate mesoderm (ALPM). Cardiogenic differentiation is initiated in the future ventricle myocardial cells by the expression of cardiac myosins (purple) at the 12-somite stage. During mid- and late-somite stages, the myocardial tissue expands by continuous cardiogenic differentiation into more lateral regions of the ALPM by the cardiogenic differentiation of future atrial myocytes (orange; venous differentiation). Whilst the endocardial cells (light green) have already migrated from the ALPM towards the mid-line, myocardial cells follow this behaviour slightly later. When the bilateral heart fields fuse at the mid-line, they form a cardiac disc structure with the endocardial cells within the hole at the centre, ventricular myocytes at the circumference and atrial myocytes at the periphery of the disc (*D*). Cardiac morphogenesis transforms the cardiac disc into a cardiac tube. The endocardium forms the inner lining of the myocardial tube. (*E*) At 28 hpf, the linear heart tube has formed, with the venous pole located at the anterior left and the arterial pole fixed at the mid-line. (*F*) Cardiogenic differentiation continues at the arterial pole, and as a result new cardiomyocytes are added to this region (purple gradient). At 36 hpf, cardiac looping has started, with a displacement of the ventricle towards the mid-line, and the constriction at the position of the AV canal is first visible (*F*). The heart tube continuous to loop and forms an S-shaped loop (*G*). Ellipsoid extracardiac pro-epicardial cells (brown) are located near the AV canal (yellow), from where they start to cover the myocardium with an epicardial layer. The pacemaker is present in the inner curvature of the atrium near the venous pole (dark green).

2. Cardiac development in zebrafish

2.1 Cardiogenic specification and differentiation

The heart is the first organ to form and function during vertebrate embryo development. Heart development begins with the specification of myocardial and endocardial progenitor cells. Cell-labelling studies have identified two pools of myocardial progenitor cells (atrial and ventricular) located in the lateral marginal zone at either side of the embryo before gastrulation starts[1] (*Figure 1A*). Atrial progenitor cells are located more ventrally in the lateral marginal zone when compared with ventricular progenitor cells.[2] The size of the myocardial progenitor pool is restricted by retinoic acid signalling.[3] In contrast to the myocardial progenitor cells, endocardial ventricular and atrial progenitors are distributed throughout the marginal zone without apparent organization.[2]

The homeobox-containing transcription factor Nkx2.5 is required for cardiogenic differentiation from *Drosophila* to humans. Its expression in zebrafish is initiated at the one- to three-somite stage. Induction of *nkx2.5* expression requires activation of the bone morphogenetic protein (Bmp) pathway, as a subset of *swirl/bmp2b* mutant embryos lack *nkx2.5* expression.[4] However, the negative feedback loop by which Nkx2.5 represses Bmp signalling activity in the anterior lateral plate mesoderm (ALPM) of mouse embryos has thus far not been studied in zebrafish.[5] Importantly, it has been suggested that regulation of *nkx2.5* expression by Bmp signalling is indirect, because overexpression of *gata5* in *swirl/bmp2b* mutant embryos restores *nkx2.5* expression.[6] Furthermore, mutant analyses have demonstrated that Nodal signalling is required for *nkx2.5* signalling and, in a similar manner to the scenario in *swirl/bmp2b* mutants, *nkx2.5* expression in Nodal mutants can be restored by ectopic expression of *gata5*.[6] Together, these results indicate that both Bmp and Nodal signalling induce *nkx2.5* expression and cardiogenic differentiation by inducing *gata5*. Indeed, *faust/gata5* mutant embryos have a reduction in *nkx2.5* expression, resulting in smaller and bilateral myocardium.[7] Finally, the basic helix–loop–helix transcription factor Hand2 is also required

for cardiogenic differentiation, because *hands off/hand2* mutant embryos have dramatically reduced myocardial tissue.[8] Hand2 acts downstream of or in parallel to Nkx2.5, as *nkx2.5* is normally expressed. Besides a cell-autonomous role for Hand2 during cardiogenic differentiation, Hand2 also has a non-cell-autonomous role during fusion of the bilateral cardiac fields by repressing fibronectin production (see below in section 2.2).[9]

During cardiogenic differentiation, expression of sarcomeric myosin genes is observed in the ALPM as early as the 14-somite stage.[10] Expression of *myosin light chain polypeptide 7* (*myl7*, formerly indicated as *cmlc2*) is initiated in few cells, with the number of cells expressing *myl7* increasing over time.[11] The myocardial cell population is regionalized in a medial to lateral direction, with the medial cells expressing *ventricle myosin heavy chain* (*vmhc*) and the lateral cells expressing *atrial myosin heavy chain* (*myh6/amhc*). This strict organization and separation of ventricular and atrial myocytes is maintained during the later stages of cardiac development, and it is currently unclear by what mechanism these two cell populations are kept separated. Interestingly, expression of the atrium-specific myosin *myh6* is initiated slightly later compared with the onset of expression of the ventricle myosin *vmhc*,[12] suggesting that the differentiation of these two myocardial cell types is initiated at different time points. Supporting this hypothesis, a developmental timing assay, making use of the different maturation times for green fluorescent protein and red fluorescent protein, demonstrated that during mid- to late somite stages there is continuous cardiogenic differentiation in the ALPM. Cardiogenic differentiation is initiated at the 12- to 15-somite stage [16 h post fertilization (hpf)] in the future ventricle cells, which are located closest to the mid-line (*Figure 1B*). This is followed by the differentiation of the more laterally located future atrial cells until the 26-somite stage (22 hpf), also referred to as venous differentiation (*Figure 1C*). After the linear heart tube has been formed (30 hpf), cardiogenic differentiation continues at the arterial pole[11] (*Figure 1F*). This late addition of cardiomyocytes to the arterial pole of the zebrafish heart resembles the addition of cardiomyocytes from the anterior or second heart field as described in amniotes.[13] The genetic pathways that regulate the addition of cardiomyocytes to the developing heart are beginning to be uncovered, although it is likely that different factors may be required for differentiation of distinct subpopulations of cardiomyocytes. For example, the late addition of new cardiomyocytes at the arterial pole depends on Fgf signalling,[11,14] best demonstrated in *ace/fgf8* mutant embryos or in embryos in which Fgf signalling has been inhibited during mid- to late-somite stages affecting the size of the ventricle.[14] As the atrium remains largely unaffected upon such treatments, it suggests that Fgf signalling is not regulating venous differentiation during mid- to late-somite stages in the ALPM. Islet-1 (Isl1) would be a good candidate for regulating the venous differentiation in the ALPM, owing to its demonstrated role in regulating cardiogenic differentiation of cells derived from the second heart field in mouse embryos. Zebrafish *isl1* is expressed in the lateral-most cells of the cardiac field.[15] Mouse and zebrafish Isl1 have different functions at the cardiac poles. In mouse embryos, Isl1 is required for the addition of cells from the second heart field to both the arterial and the venous pole, whereas *isl1* mutant zebrafish embryos have a significant although moderate reduction in the cardiogenic differentiation only at the venous pole. A possible candidate for regulating the venous differentiation in the lateral regions of the ALPM is Bmp signalling. Bmp ligands are known for their cardiogenic potential.[16] In addition, *laf/alk8* mutant embryos, containing a mutation in one of the Bmp type I receptors, develop hearts with a reduced atrium, while the size of the ventricle remains unaffected.[17,18] Analysis of temporal and spatial (in)activation of Bmp signalling in the ALPM during cardiogenic differentiation, together with temporal rescue of the *laf/alk8* phenotype should allow us to address this issue. Together, these data show that a number of spatially restricted factors are required in concert to generate continuous regionalized cardiac differentiation.

2.2 Migration and fusion of the bilateral heart fields

During gastrulation and early somite stages, the cardiac progenitor cells converge towards the mid-line to reach their destination in the ALPM, which requires *grinch* (*Figure 1B*). The *grinch* mutant, identified in a forward genetic screen, exhibits a reduced number of myocardial cells. *Grinch*, which encodes a G-coupled Apelin receptor (*agtrl1b*), is expressed in the marginal zone and ALPM, while its ligand, Apelin, is expressed in the mid-line.[19,20] Cell-labelling experiments demonstrated that Agtrl1b and Apelin are required to enable migration of the bilateral pools of myocardial progenitor cells towards the mid-line, where they receive the appropriate signals to induce their cardiogenic differentiation.

During mid- and late-somite stages, cardiogenic differentiation and heart morphogenesis occur simultaneously, often making it difficult to attribute the function of a gene specifically to one of these processes. In some cases, the identified factor plays a role in both processes; for example, in the case of Hand2.[9] Independent from its role in regulating cardiogenic differentiation, Hand2 is also required for the movement of the cardiac fields towards the embryonic mid-line by regulating fibronectin levels. Analysis of cardiac fusion in *natter* mutant embryos revealed that the two bilateral heart fields fail to migrate towards the mid-line, resulting in cardia bifida.[21] The *natter* mutants harbour an inactivating mutation in *fibronectin*, demonstrating an essential role for extracellular matrix fibronectin during this process.[22] Fibronectin is deposited in a thin layer at the interface of the myocardial tissue and the yolk syncytial layer, an extra-embryonic tissue also required for myocardial migration.[23] Indeed, analyses of a number of mutants have helped to uncover the role of this structure in promoting cardiomyocyte migration. Expression of the spinster homologue *two-of-hearts/toh* in the yolk syncytial layer regulates the transport of sphingosine 1-phosphate (s1p) from the yolk to the embryonic tissue.[24,25] In the embryonic tissue, the s1p receptor *miles apart* (*mil/s1p2*) is required for proper migration of the cardiac field towards the mid-line. Furthermore, in *mil/s1p2* mutant embryos, fibronectin levels are reduced, suggesting that s1p from the yolk regulates embryonic fibronectin levels to allow proper migration of the bilateral heart fields to the mid-line.[24,26] Besides s1p, the extra-embryonic syndecan 2 (Sdc2), a heparan sulfate proteoglycan, is also required for migration of the bilateral heart fields towards the mid-line.[27] Knock-down of *sdc2* in the extra-embryonic yolk results in a reduction of fibronectin at the yolk syncytial layer–embryo interface and subsequently affects the polarity of the myocardial cells.

2.3 Formation of the endocardium

The endocardium is derived from a distinct region in the ALPM that also gives rise to haematopoietic cells of the primitive myeloid lineage.[28] Using transgenic zebrafish expressing different fluorescent

proteins in the endothelium and myocardium and four-dimensional confocal microscopy, it was shown that endocardial cells migrate towards the mid-line slightly before the myocardial cells do so.[28] After the bilateral myocardial heart fields have migrated in a coherent and medial direction towards the mid-line, the peripherally localized cardiomyocytes change their direction of movement in the anterior and posterior direction. At this point, the myocardial cells at the posterior and anterior ends of the myocardial sheet move inward towards the endocardial cells already located at the mid-line and thereby establish the initial circumference of the nascent heart tube.[29] Once the bilateral heart fields have fused at the mid-line, the cardiac tissue forms a disc, in which the future ventricle cells are located at the circumference, the endocardial cells at the centre, and the future atrial cells at the periphery (*Figures 1D* and *2A*).

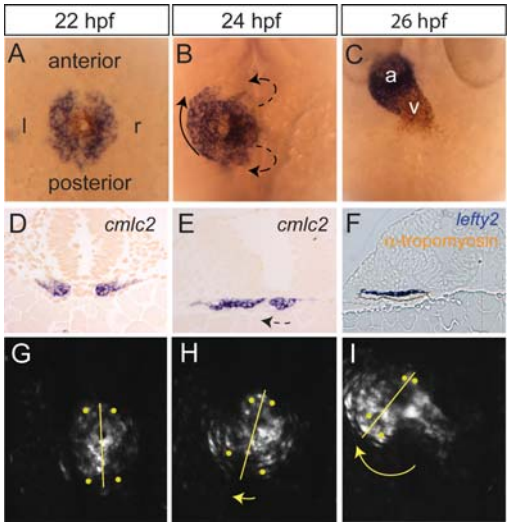

Figure 2 Heart morphogenesis. Dorsal views (*A–C*, *G*, and *H*) and cross-sections (*D–F*) of the heart field are shown at three different stages: 23 somites or 20.5 hpf (*A*,*D*, and *G*), 25 somites or 22 hpf (*B*,*E*, and *H*) and 24 hpf (*C*,*F*, and *I*). (*A–C*) Double-labelling *in situ* hybridization with *ventricle myosin heavy chain* (*vmhc*, red) and *atrial myosin heavy chain* (*amhc*/*myh6*, blue) probes. (*D–F*) Cross-sections after *in situ* hybridization with a *cmlc2*/*myl7* probe (*D* and *E*) or a *lefty2* probe (blue) with an anti-tropomyosin antibody (brown). (*G–I*) Projections of confocal stacks made from a living *Tg(cmlc2:eGFP)* embryo. Tracking of individual myocytes was performed, and four examples are shown as yellow dots. After fusion of the bilateral heart fields, a cardiac disc is formed, which is located ventrally to the neural tube and endoderm (*A*,*D*, and *G*). The future ventricle cells (blue cells in *A* and *B*) are located in the central region of the disc, where they start to form a cone due to an involution of the tissue at the right side (dashed arrows in *B* and *E*). The future atrial cells (blue in *A* and *B*) are located at the periphery of the disc. These cells are displaced towards the anterior left and cause the future venous pole to rotate in a clockwise direction (arrows in *B*, *H*, and *I*). Owing to the involution process and rotation processes, the left cardiac field will form the dorsal wall of the heart tube, indicated by *lefty2* expression in *F*. The myocytes derived from the right cardiac field will form the ventral wall of the cardiac tube (*lefty*-negative cells in *F*). When the myocardial tube has formed, it will extent anteriorly by a thus far unidentified process.

2.4 Formation and rotation of the myocardial tube

Up to disc stage, the heart morphogenesis process can be regarded as symmetrical, because there are no morphological differences between the left and right cardiac fields (*Figure 2A*,*C*,*G*). However, once the cardiac disc has formed the myocardial tissue originating from the right cardiac field involutes ventrally and moves towards the anterior/left (*Figure 2B*,*E*,*H*). As a consequence, the original left–right organization of the cardiac disc is transferred into a dorsal–ventral organization of the cardiac tube (*Figure 2F*). Endocardial cells that were located ventral to the myocardial disc will be positioned within the lumen of the cardiac tube.[28,30,31] Four-dimensional confocal miscroscopy combined with automated cell tracking revealed that simultaneous with right-field involution, the myocardial disc rotates in a clockwise direction (*Figure 2G–I*).[30,32,33] As a consequence of asymmetric involution and rotation of the myocardial tissue, a cardiac tube is formed, with its venous pole located at the left side and its arterial pole still at the mid-line (*Figures 1E* and *2C*). This directed migration of the myocardial cells requires the extracellular matrix component hyaluronic acid, which is a glycosaminoglycan produced by *hyaluronan synthase 2* (*has2*).[32] Concomitantly with the translocation of the venous pole towards the left side of the embryo, the heart tube also extends during this time (*Figure 2C*). The polarity and epithelial organization of myocardial cells is essential for the extension of the cardiac tube, as loss of cell polarity proteins causes an arrest of cardiac morphogenesis at the heart disc stage.[31,34,35]

Nodal and Bmp, which belong to the transforming growth factor-β (Tgf-β) superfamily of growth factors, are asymmetrically expressed in the ALPM and direct asymmetric heart morphogenesis. Disruption of Nodal signalling within the heart field by the knockdown of *southpaw* (*spaw*)/*nodal*, or randomization of *spaw*/*nodal* expression due to immotile cilia disrupting nodal flow in *switch hitter* mutants resulted in a randomization in the direction of heart morphogenesis.[30,31] Supporting these findings, the rotational movement of the cardiac disc is impaired in embryos lacking the Nodal co-receptor *one-eyed pinhead*/*egf-cfc*.[33] Prior to asymmetric involution and rotation of the cardiac disc, *bmp4* is asymmetrically expressed in the ALPM, with elevated levels of expression on the left side.[36] Also, enhanced Bmp activity in the left ALPM was observed using an antibody recognizing phosphorylated Smad1,5,8 proteins, which are downstream mediators of Bmp signalling.[32] Blocking Bmp signalling at the cardiac disc stage by over-expression of the Bmp inhibitor Noggin, using a heat shock-inducible transgenic line, prevented the leftward movement and rotation of the myocardial tissue.[18,32] As asymmetric *bmp4* expression is disrupted in mutants lacking the Nodal-related genes *cyclops* or *southpaw* or the transcription factor *schmalspur*/*foxh1*, which activates Nodal target genes, it is conceivable that the asymmetric Bmp activation is regulated by unilateral Nodal activity in the left ALPM.[18,36] Experiments with ectopic activation of Nodal and Bmp signalling in the left or right ALPM in Nodal and Bmp signalling mutants should address the inter-relationship between these two pathways during asymmetric heart morphogenesis. Cardiac extension is still observed in embryos in which Nodal or Bmp signalling has been disrupted, suggesting that heart tube extension and leftward displacement are separately regulated.

2.5 Cardiac looping and chamber ballooning

After the linear heart tube has formed, it bends towards the right side. The bending is best described as a displacement of the future ventricle so that it will be positioned at the right side of the embryonic mid-line, while the future atrium remains positioned at the left side of the mid-line (*Figure 1F*). Little is known regarding the cellular processes that facilitate bending of the heart tube and the underlying molecular mechanisms. Interestingly, the direction of cardiac looping is dictated by the preceeding direction of cardiac displacement. For example, heart tubes that extend to the right side of the embryo will ultimately loop in the reverse direction.[36] In addition, the degree and direction of rotation of the cardiac disc as described above is correlated with the degree and direction of the cardiac displacement.[30,32] Importantly, not all parts of the cardiac tube rotate equally. Strong rotation is observed in the future venous pole, while very little rotation is observed in the future arterial pole.[32] The different rates of heart tube rotation result in torsion of the cardiac tube, and biomechanical models have proposed that rotation at one of the poles of the heart tube is sufficient to drive dextral looping.[37,38] The use of forward genetic screens to identify mutants with specific defects in cardiac displacement and looping will help to identify critical regulators of the looping process in the future.

During cardiac looping, the ventricle and atrium become morphologically distinguishable as bean-shaped chambers with a distinct outer curvature and inner curvature, through a process referred to as chamber ballooning.[39] Expression of *natriuretic peptide type A* (*nppa*) marks the outer curvature myocardium in both mouse and fish hearts and is first detected at 28 hpf.[40] During ventricular chamber emergence, regionally confined cell shape changes are observed in the outer curvature. The outer curvature cells are typically oriented with their long axes perpendicular to the arterial–venous axis, suggesting that cardiomyocyte elongation plays a substantial role in creating the characteristic convex shape of the outer curvature.[40] Furthermore, blood flow through the developing ventricle promotes cardiomyocyte elongation and thereby encourages establishment of the outer curvature, while intrinsic contractility restricts cardiomyocyte elongation, preventing cardiac dilatation.[40]

2.6 Atrioventricular valves

Cardiac valves are critical for proper functioning of the vertebrate heart, and malformation of the valves underlies many forms of human congenital and adult-onset heart diseases, such as aortic or pulmonary valve stenosis, bicuspid aortic valve, mitral valve prolapsed, and Epstein's anomaly. The presence of valves between the ventricle and atrium prevents blood from flowing back from the ventricle to the atrium when the ventricle contracts. Cardiac valve formation has been most extensively studied in chick and mouse embryos (for recent review see Combs and Yutzey[41]). In amniotes, valvulogenesis starts with the formation of an endocardial cushion in the atrioventricular (AV) canal. Endocardial cells overlying this local swelling of the cardiac jelly (extracellular matrix located between the myocardium and endocardium) receive signals from the AV canal myocardium to undergo an epithelial-to-mesenchymal transition. Delaminated endocardial cells migrate into the cardiac jelly and fill these local swellings by proliferation. In the zebrafish heart, the first signs of endocardial transdifferentiation are observed at 36 hpf, when squamous endocardial cells located in the future AV canal become cuboidal and start to express the cell adhesion molecule Dm-grasp.[42] By 48 hpf, endocardial cells lining the AV canal form a single layer of cuboidal cells that express Dm-grasp, in contrast to the squamous Dm-grasp-negative endocardial cells lining the chamber myocardium. At this stage, the Dm-grasp-positive endocardial cells form cellular protrusions that extend into the cardiac jelly. Recent studies employing live high-speed imaging using single-plane illumination microscopy revealed that although the endocardial cells form a multicellular cushion structure, these endocardial cells remain epithelial and do not delaminate in the manner described for endocardial cushion cells in the amniote heart.[43] These results suggest that the endocardial cushions in zebrafish do not form by epithelial-to-mesenchymal transition, but instead by invagination of the endocardial cells.[44] Although the mechanism by which the endocardial cushions arise in zebrafish appears to differ from amniotes, the molecular signals regulating the process have been conserved. As in amniotes, Notch, NFAT, ErbB and Tgf-β signalling are required for cardiac valve formation in zebrafish.[42–45] In addition to the above-mentioned regulators, ectopic Wnt/β-catenin signalling induces endocardial proliferation and the expression of *has2*, which is required for epithelial-to-mesenchymal transition in the endocardial cells of the mouse AV canal.[46] To gain a better understanding of valve formation in amniotes and zebrafish, it will be important to investigate the cellular processes that are regulated by these signalling pathways.

By 105 hpf, the endocardial cushions have enlarged and differentiated into valve leaflets. The AV valve leaflets, which are composed of two layers of cells separated by a layer of fibronectin-containing extracellular matrix, extend into the lumen of the ventricle.[42,44] In addition to genetic factors, environmental factors also influence valve formation. In mutant embryos that display defects in early myocardial function, such as *silent heart/sil*, which lack a heartbeat due to mutation of *cardiac troponin T* (*tnnt2*), or *cardiofunk/cfk*, encoding a novel sarcomeric actin expressed in the embryonic myocardium, cardiac valve formation is impaired.[47] Furthermore, blood flow through the heart results in endothelial sheer stress, which is required for correct cardiac valve formation.[48] Oscillating blood flow through the AV canal induces the expression of *klf2*, a shear-reponsive gene, in the endocardial cushion cells. *klf2* acts upstream of Notch1 and is required for the cell shape changes and invagination process observed in the endocardial cushions.

2.7 Conduction system

Cardiomyocyte contraction is first observed when the linear heart tube is being formed. Initial cardiomyocyte contractions are irregular and unco-ordinated, but show a co-ordinated pattern once the heart tube has formed. Contractions are initiated at the venous pole. Owing to slow conduction of the impulse throughout the heart tube, these contractions are initially peristaltic. As the heart develops, the rate of contractions increases and changes from a peristaltic contraction into a sequential contraction of the atrium and ventricle. In 1949, Patten first observed that in the chick heart the most recent cardiomyocytes added to the venous pole possess an intrinsically higher contraction rate than the part of the cardiac tube already formed.[49] As the part of the cardiac tube with the highest contraction rate at any given phase of development sets the rate for the entire heart, he concluded that the pacemaker is always located in the part of the heart that was added most recently. By expressing light-gated ion channels in zebrafish, the pacemaker or sino-atrial node was recently located at the inner curvature of the venous pole, the

region that is added last to the cardiac tube (*Figure 1G*).[11,50] Interestingly, the cardiomyocytes added to the venous pole express *isl1*, which encodes a LIM-homeodomain-containing transcription factor also expressed in motorneurons. Zebrafish *isl1* mutant embryos display bradycardia and arrhythmias, suggesting a role for Islet-1 in the pacemaking cells.[11]

During cardiac looping (36–48 hpf), a slowing of the electrical pulse is observed in the region of the AV canal, also referred to as AV delay, resulting in a contraction delay at the AV boundary.[51,52] This AV delay coincides with the transition from a peristaltic contraction of the heart tube into a more efficient sequential contraction of the atrium followed by the ventricle. Cardiomyocytes are positioned circumferentially around the AV canal, suggesting that morphological differences between cardiomyocytes of the AV canal and the chamber myocardium contribute to the AV delay. In *cloche* mutants, which lack all endothelium including the endocardium, the conduction delay in AV canal myocardium is not observed.[51] This observation suggests that endocardium-derived signals cause the overlying myocardium to differentiate into slow- and fast-conducting myocardium. Knock-down of *notch1b* or *neuregulin*, which are expressed in the endocardium, also results in a loss of the AV conduction delay in the AV canal myocardium.[51] The role of endocardial Neuregulin seems conserved in other species, because in mouse embryos *Neuregulin* is expressed in the endocardium and can promote the formation of the cardiac conduction system.[53,54] Several signalling pathways and transcription factors expressed in the AV canal myocardium have been identified that are required to maintain a primitive and slow-conducting AV canal myocardium. For instance, expression of the T-box transcription factor *tbx2b* in the AV canal myocardium is required for the observed AV conduction delay.[55] Analogous to its activity in the mouse heart, Tbx2b prevents the expression of *nppa*, a marker for cells belonging to the fast-conducting outer curvature of the chamber myocardium. Several factors have been identified that regulate *tbx2* expression in the AV canal. Zebrafish *bmp4* is expressed in the AV canal, and Bmp signalling activity regulates expression of the *tbx2b* in the AV canal myocardium.[55,56] Furthermore, in *apc* mutant embryos, in which Wnt signalling is enhanced, *bmp4* expression is expanded, suggesting that Wnt signalling in the myocardium regulates *bmp4* and *tbx2* expression in the AV canal. Indeed, myocardial contraction remains slow, without any obvious AV delay, in *apc* mutant hearts.[56] Expression of *tbx2b* in the AV canal is also controlled by the forkhead transcription factor *foxn4*, and consequently loss of *foxn4* leads to a loss of the AV conduction delay.[55]

2.8 Epicardium

During linear heart tube and looping stages, the heart wall is composed of only two cellular layers, the myocardium and endocardium. A third cellular layer, the epicardium, which covers the outside of the myocardium, develops from an extracardiac population of cells called the pro-epicardium. The pro-epicardium can be distinguished morphologically at 48 hpf as a group of spherical cells located in close proximity of the ventral wall of the looped heart (*Figure 1G*). At 72 hpf, it has increased in size and starts to spreads over the myocardial surface of the looped heart.[57,58] Molecularly, the pro-epicardium is characterized by the onset of expression of *wt1*, *tbx18* and *tcf21* at 40–48 hpf. Specification of the pro-epicardium requires a Bmp ligand, which is not derived from the liver bud as was previously assumed.[58] *bmp4* is expressed in the myocardium and is required for pro-epicardium specification, suggesting that a cardiac Bmp signal induces pro-epicardium specification. Independently from Bmp signalling, *tbx5* expression in the lateral plate mesoderm during the early somite stages is also required for pro-epicardium specification.[58] Although it is well described in zebrafish, chick and mouse embryos that extracardiac pro-epicardial cells form a bridge to the myocardium and spread onto it, thus far the instructive molecular signals driving this process are largely unknown.

In the adult heart, the epicardium is a squamous epithelium, which forms a smooth surface covering the entire heart wall. Upon injury of the heart, for example by dissection of the apex of the ventricle, the zebrafish epicardium responds by elevating expression of the embryonic epicardial markers *tbx18* and *raldh2*. This suggests that an embryonic gene expression programme in the epicardium is activated in response to injury.[59] This activation starts throughout the entire ventricle and gradually becomes localized to the apex. The activated epicardial cells proliferate and undergo epithelial-to-mesenchymal transition, which requires the platelet-derived growth factor.[60] The developmentally activated epicardial cells then invade the newly formed myocardium at the site of injury and create a dense vascular network that is likely to encourage regeneration.[59,60]

3. Zebrafish to model human congenital and acquired cardiac diseases

3.1 Congenital heart defects

Currently, approaches for discovering monogenetic factors involved in human congenital heart defects (CHDs) include pedigree studies and candidate screens. Classical forward genetic human pedigree studies have thus far provided limited insight into the genetic causes of CHDs. More recently, reverse genetic approaches have been used to identify single nucleotide polymorphisms (SNPs) in candidate genes. In such an approach, candidate genes are typically selected from pre-existing knowledge obtained from basic scientific studies using various model organisms.[61–63] This knowledge can come from the zebrafish model, as demonstrated by a study in which mutations where identified in the Nodal co-receptor EGF-CFC/CRYPTIC in patients with severe congenital visceral laterality defects.[64] In a recent candidate screen, which consisted of sequencing the coding regions (exons) of 32 candidate genes of 190 CHD patients, a number of novel and potential genetic determinants of atrioventricular valve and septum (AVS) defects were identified.[63] By using two different algorithms, which can predict the affect of the mutation on protein function, 11 patient-specific coding SNPs (cSNPs) were identified that are likely to be damaging. One such cSNP was identified in *GATA4*, for which a causal relation with AVS defect was demonstrated in other studies.[65] The 10 remaining cSNPs were found in genes not previously linked to AVS defects in human subjects such as *ALK2*, *ALK3*, *APC*, *ECE2*, *EGFR*, *ERBB3*, *FOXP1*, *ADAM19*, and *UGDH*. Expression of the patient-specific *ALK2* L343P variant in zebrafish showed that this results in a malformation of the AV canal and compromised expression of endocardial cushion markers. Additionally, *in vivo* studies demonstrated either reduced or dominant-negative activity of the disease-related *ALK2* (a BMP receptor) variants.[63,66] A role for *UGDH*, encoding a UDP-glucose dehydrogenase, in cardiac valve formation was demonstrated

first in zebrafish. The *jekyll* mutant, harbouring a loss-of-function mutation in the zebrafish *ugdh* gene, does not form proper AV valves.[21,67] The complex process of AV valve and septum formation, with its multifaceted stages and various signalling pathways involved, would indicate that aberrant AVS development indeed can occur at numerous steps in the process. Array comparative genomic hybridization and next-generation sequencing are new technologies with promising possibilities for detecting genomic alterations in CHD patients.[68] An advantage of the candidate gene approach is that genetic variants can be identified relatively rapidly and in small pedigrees or even single individuals. Next-generation exome or whole-genome sequencing will change the landscape of personalized diagnostics, through cataloguing all genomic variations of a single affected individual.[69]

The major short-coming of candidate screening, array comparative genomic hybridization, and next-generation sequencing still remains that from any variant discovered within an affected individual or population it remains unknown whether the variant is potentially disease causing or not. As a consequence, testing the potential *in vivo* effect of the identified variants is important though challenging. In cases where genotype–phenotype information on the affected families is lacking or not conclusive, the zebrafish model has proved itself as a useful tool in such studies.[63,64]

3.2 Cardiomyopathies

Human cardiomyopathies are diseases that primarily affect the myocardium. The two most prevalent forms are dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). DCM is a syndrome characterized by enlargement of one or both ventricles of the heart, accompanied by diminished myocardial contractility. HCM is defined as thickening of the myocardium in the left and/or right ventricle in the absence of any other diseases that cause myocardial hypertrophy, such as high blood pressure or storage diseases. HCM is considered to be multifactorial with a strong genetic influence, while DCM has a genetic contribution in at least 30% of all cases. DCM is associated with mutations in genes encoding cytoskeletal or contractile proteins. One mechanism underlying the development of DCM is defective cardiac stretch sensing.[70] Several zebrafish mutants have been identified that display heart phenotypes resembling the features of human cardiomyopathies, and their characterization has provided a better understanding of the human disease. In addition, identification of the genes affected in these mutants has provided novel candidate genes that allow further characterization of the genetics of human cardiomyopathies (summarized in *Table 1*).

In a large-scale screen for recessive lethal mutations due to cardiac dysfunction, the *silent heart/sil* and *pickwick/pik* mutants were identified.[21] The hearts of homozygous *pik* and *sil* embryos develop normally but are poorly contractile from the first beat. Positional cloning of two *sil* alleles revealed inactivating mutations in the *tnnt2* gene encoding cardiac troponin T.[71] Mutations in human *TNNT2* cause DCM and familial HCM, the leading cause of sudden death in young athletes, making the *sil/tnnt2* mutant an excellent model to study the role of Tnnt2 in cardiac function and disease. Positional cloning of the *pik* locus revealed a causative mutation in the *ttn* gene encoding the large sarcomeric titin protein.[72] The titin protein spans the half-sarcomere from Z-disc to M-band and is required for proper sarcomere assembly, as well as confering elasticity on the sarcomeres. Cardiomyocytes devoid of a functional titin protein are elongated and thin when transplanted into a wild-type heart, resembling a DCM phenotype in zebrafish.[72] Notably, in a large human pedigree a 2 bp insertion in the *TTN* gene was identified, causing a frameshift and subsequent truncation of titin, which was strongly associated with autosomal dominant DCM in the studied family.[73]

The same large-scale screen in which the *sil* and *pik* mutants were identified also led to the identification of the *main squeeze/msq* mutant, which displays ventricle dysfunction starting at 60–70 hpf.[21] In an independent screen, the *lost-contact/loc* mutant was identified, which is allelic with the *msq* mutant.[61] Positional cloning of the *loc* and *msq* alleles revealed a premature stop mutation and a mis-sense mutation, respectively, in the gene encoding integrin-linked kinase (Ilk).[61,74] Integrins interact with laminin

Table 1 Zebrafish cardiac mutants with related human disease

Zebrafish gene name	Zebrafish loss-of-function phenotype	Human gene name	Related human heart disease	Reference
one-eyed-pinhead/egf-cfc	Reduced mesendoderm differentiation, visceral laterality defects and cardia bifida	*CRYPTIC*	Dextrocardia, transposition of the great arteries, double-outlet-right-ventricle, AVS defect	6,64,86
lost-a-fin/alk8	Unlooped heart with reduced atrium	*ALK2*	AVS defect, mitral valve malformation	36,17,18,63,66
jekyll/ugdh	AV valves fail to form	*UGDH*	AVS defect	63,67
apc	Unlooped heart with hyperplastic endocardial cushions	*APC*	AVS defect	46,63
silent-heart/tnnt2	Poorly contractile ventricle and atrium	*TNNT2*	DCM and HCM	71
pickwick/ttn	Poorly contractile ventricle and atrium	*TTN*	DCM	72,73
lost-contact/main-squeeze/ilk	Reduced contractility and aberrant cardiomyocyte shape, dilated and reptured blood vessels	*ILK*	DCM	21,61,74
laminin alpha 4	Genetic interaction with lost-contact/ilk	*LAMA4*	DCM	61
nexilin/nexn	Disturbed sarcomere integrity and dilated heart	*NEXN*	DCM	62,75
chap	Disturbed sarcomere integrity and dilated heart	*CHAP*	Unknown	76

present in the extracellular matrix, and genetic studies in zebrafish showed that *loc/ilk* interacts genetically with *lama4*.[61] Cardiomyocytes devoid of a functional integrin-linked kinase protein appear stretched and thin when transplanted into a wild-type heart. Based on these results, mutations in human *ILK* and *LAMA4* were identified in patients with DCM.[61]

Within the sarcomeres, the myofilaments are anchored to the Z-disc, the mechanical integration site of heart muscle cells. Several key molecules that enable the Z-disc persistently to withstand the extreme mechanical forces during muscle contraction have been identified, such as MLP, T-Cap, ILK, α-actinin, and integrins. Recently, several novel Z-disc proteins have been identified, whose function has been studied *in vitro* and *in vivo* using zebrafish. Loss of nexilin (encoded by *nexn*) in zebrafish led to perturbed Z-disc stability and heart failure.[62] To evaluate the role of nexilin in human heart failure, two independent candidate screens were performed on individuals with either DCM or HCM, and several mutations in *NEXN* were found.[62,75] Another new Z-disc protein, encoded by *Chap* (cytoskeletal heart-enriched actin-associated protein), is expressed in differentiating heart and skeletal muscle.[76] Interestingly, in addition to its sarcomeric localization, Chap is also able to translocate to the nucleus. Knockdown of *chap* in zebrafish results in aberrant cardiac and skeletal muscle development and function.

Many regulatory elements that drive expression of cardiac genes are being identified using chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-Seq). ChIP-Seq is used to identify the *cis*-acting targets of DNA-associated proteins. Recently, a ChIP-Seq-based genome-wide study aimed at discovering human heart enhancers was published.[77] Validation of these enhancers *in vivo* in mouse and zebrafish resulted in a validation rate of 62%, demonstrating the usefulness of the zebrafish model in verification of results obtained by large-scale genome projects.

3.3 Gene targeting of novel cardiac genes

Various studies have described the isolation of novel proteins that are expressed in cardiac muscle with a previously unknown function.[62,76,78–80] Whilst *in vitro* experiments provide valuable information with respect to the cellular distribution or effects on muscle differentiation of such novel proteins, essential information about their *in vivo* role during cardiac development or function requires loss-of-function studies in a whole organism. Currently, there are several technologies available to perform loss-of-function studies in zebrafish (reviewed by Skromne and Prince[81]). Here, I will list and explain briefly the most commonly used technologies for reverse genetics, also known as the 'phenotype-driven approach'. Protein activity can be perturbed using small chemicals that are water soluble and are efficiently taken up by the embryo when added to the embryo medium. This approach can work well for proteins with enzyme activities, such as kinases, or to prevent protein–protein interactions. The advantage of a chemical approach is that the chemical can be added to the medium at the relevant stages to study cardiac development or cardiac function, preventing earlier developmental defects. Additionally, chemicals can also be washed out from the embryo, resulting in a tight temporal control. A drawback of the chemical approach is the limited availability of chemicals that block specific protein function, making this technology suitable only for well-characterized proteins. In addition, the specificity of the chemical for its target protein(s), depending on how numerous they are, is often a problem. Thus far, the most frequently used technology to perturb the function of uncharacterized proteins is the use of antisense morpholino oligonucleotides.[82] Morpholino oligonucleotides are short chains of about 25 modified nucleotides, which make them resistant to RNAse degradation. Morpholinos do not degrade their RNA targets, but instead act via a steric blocking mechanism. Depending on the oligo sequence, they can either block translation initiation, by targeting the 5′ UTR through the first 25 bases of coding sequence, or modify pre-RNA splicing, by targeting splice junctions. The oligonucleotides are injected into the oocyte directly after fertilization and can be effective up to 3–4 days post fertilization. Although the oligonucleotides are designed to target the gene of interest exclusively, they do not always result in an efficient knock-down of the gene of interest and are also prone to off-target effects. To address whether the observed phenotypes after morpholino injection are due to knock-down of the gene of interest, proper control experiments must be performed.[83] As zebrafish have a partly duplicated genome, it often contains two paralogous genes when the mouse or human genome contains a single gene. As a consequence, one would need to knock down both genes to observe the complete loss-of-function phenotype. To prevent off-target effects or to study cardiac gene function at stages beyond the 3- to 4-day-old embryo, it is possible to use target-selected mutagenesis, which comprises random chemical mutagenesis, followed by screening for mutations in target genes.[84] This approach results in a stable mutant in the gene of interest, resolving the variability and off-target problems associated with the previously described technologies. Its disadvantage is the requirement for a large fish facility and the availability of a high-throughput screening platform, which limits its application to larger research groups or centres. More recently, the use of zinc finger nucleases (ZFNS) has been introduced as a new technology for gene targeting in zebrafish (reviewed by Urnov[85]). The ZFN technology is based on the combined use of sequence-dependent DNA-binding domains of a specific class of eukaryotic transcription factors, zinc finger proteins, together with the nuclease domain of the FokI restriction enzyme. Owing to their modular approach, ZFNs have the advantages of DNA-binding specificity and the flexibility of zinc finger proteins and can be targeted to specific sites within the genome. ZFN technology combines the advantages of the specificity of gene targeting with the inheritability of the generated gene disruption. Its drawbacks are the limited chance of finding ZFN targets sites in small genes and the amount of work that is required to select the proper ZFNs that efficiently recognize and cleave the target sequence. Thus far, the ZFN technology has not been used to study the loss-of-function phenotype of novel cardiac genes.

4. Conclusion

In the last decade, the zebrafish model has developed into a very powerful model to study cardiac development. Owing to the efforts of forward genetic screens, many novel factors and regulatory mechanisms have been identified that have essential roles during cardiogenic specification and differentiation, migration of cardiac progenitor cells, heart tube morphogenesis, and cardiac function. By developing new and more specialized assays, forward genetic screen efforts will continue to contribute new insights to the field of cardiac development in the years to come. In addition, the growing toolbox that allows specific gene disruptions in combination with tissue-specific and inducible expression systems will make the

zebrafish for many researchers the preferred model organism to study their gene of interest. More recently, the zebrafish has been used to study mechanisms leading to human cardiac diseases and to model human congenital and acquired cardiac diseases. Predictably, this field will grow rapidly in the coming years owing to the increase in sequencing efforts, the growing interest in cardiac diseases, and the improved availability of the zebrafish model for clinical and basic researchers interested in studying cardiac diseases.

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